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Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland

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Abstract

Symbiotic interactions are thought to play a key role in ecosystems. Empirical evidence for the impact of symbiotic bacteria on plant communities is, however, extremely scarce because of experimental constraints. Here, in three complementary experiments, we show that nitrogen-fixing rhizobia bacteria act as a determinant of plant community structure and diversity. Grassland microcosms inoculated with a mixture of rhizobia had a higher above-ground plant productivity (+35%), contained more nitrogen (+85%) and had significant higher community evenness (+34%) than control microcosms without rhizobia. Moreover, three of the four studied legume species required rhizobia to successfully coexist with other plant species. In contrast, the growth and survival of three grass and five forb species were not affected by the presence or absence of rhizobia. Finally, our results also showed that the legume species largely relied on symbiotically fixed nitrogen, both in the field and in the microcosms. This indicates that results in the microcosms are indicative for processes occurring in the field. It is concluded that symbiotic interactions between plants and prokaryotes can contribute to plant productivity, plant community structure and acquisition of limiting resources in legume-rich grassland communities.

Introduction

One of the major goals in plant ecology is to search for the mechanisms that determine plant community structure and affect ecosystem processes. Factors such as nutrient availability, climate, history, herbivory and plant–soil feedback are known to shape plant communities (Tilman, 1988; Loreau *et al.*, 2001; Grime, 2001; Klironomos, 2002). Increasingly, it is being recognized that mutualistic interactions between plants and microbes also play a key role in ecosystems (Smith & Read, 1997; Clay & Holah, 1999; Bruno *et al.*, 2003). An important group of mutualists are the nitrogen-fixing bacterial associates of legumes (Turkington *et al.*, 1988; Sprent, 2001). These bacteria, collectively called rhizobia, induce root nodules and fix atmospheric nitrogen into ammonium that is delivered to their leguminous hosts. Rhizobia form symbiotic relationships with an estimated 15 000 legume species (Sprent, 2001). This estimate is based on the proportion of legumes in the three subfamilies that were examined for nodulation, and which were nodulated, multiplied by the total number of legumes in each subfamily. The legume–rhizobia symbiosis is important because nitrogen is one of the main elements that limits plant productivity in natural ecosystems. The importance of rhizobia in species-poor agricultural systems has been well studied. This is not surprising as several economically important crops (e.g. soybean, alfalfa, clover, pea) benefit from symbiotically fixed nitrogen and obtain a higher yield (Marschner, 1995). In contrast, the role of rhizobia in natural communities is poorly understood and empirical evidence is extremely scarce (Parker, 1995; van der Heijden & Cornelissen, 2002).

Several recent studies have shown that legumes play a key role in species-rich grassland by increasing plant productivity and nitrogen capture (Tilman *et al.*, 1997; Hector *et al.*, 1999; Spehn *et al.*, 2002; Mulder *et al.*, 2002; Hooper & Dukes, 2004). Rhizobia are probably responsible for this, even though it is unclear to what extent and by what mechanisms. In view of this, and because rhizobia have been proposed as keystone species (Wardle, 2002) and ecosystem engineers (Crooks, 2002), it is surprising that the influence of rhizobia on complex plant communities consisting of

forbs, grasses and legumes has never been experimentally tested. In order to study the potential role of rhizobia in natural ecosystems, controlled experiments are required, in which it is possible to manipulate the presence of rhizobia. This is difficult because rhizobia are not amenable to field manipulation, as the bacteria are usually already present in the field and disperse easily through air and soil causing contamination in control plots. Hence, it is necessary to perform experiments under controlled conditions in which it is possible to reduce the probability of contamination.

There are several ways in which rhizobia can influence plant communities. By providing nitrogen and stimulating legume growth, rhizobia can alter competitive interactions. Legumes may out-compete other plants when rhizobia are present (e.g. by reducing light intensity or decreasing the availability of nutrients). Rhizobia may also facilitate the growth of other plant species by increasing nitrogen availability in the soil. This is possible when nitrogen transfer from legumes to nonlegumes occurs or when legume roots or root nodules decompose (Heichel, 1987; Mårtensson *et al.*, 1998). Intercropping of legumes and nonlegumes, a common practice in agriculture, is based on this principle (Trenbath, 1974; Vandermeer, 1989). A further option is that rhizobia only influence legume growth and that coexisting plant species are not affected. In this scenario, niche separation and complementary resource use occur: the legumes utilize atmospheric nitrogen, whereas other plants acquire soil nitrogen. This situation is most probably typical of systems in which plant productivity is limited by the availability of nitrogen and in which competition for other resources is less important. Finally, some studies have indicated that rhizobia directly promote the growth of various nonhosts (Yanni *et al.*, 1997; Antoun *et al.*, 1998). This is another way in which rhizobia may influence plant community structure, even though its significance in natural vegetation is unclear.

In this study, we addressed the following questions.

(1) Do rhizobia affect plant community composition, productivity and nitrogen availability under sterile conditions? In order to test this, we established 16 experimental grassland communities to which we added an inoculum of rhizobia isolates or an autoclaved inoculum as a control (see Experiment 1).

(2) Do rhizobia affect plant community composition, productivity and nitrogen availability under more realistic conditions? We conducted a similar experiment, but used communities to which we added other bacteria and fungi from natural soils. We also ran this experiment for a longer period of time (8 months), and clipped the plants to simulate grazing (see Experiment 2). To track nitrogen dynamics, we labelled the soil with the stable nitrogen isotope ^{15}N, and assessed the variation in ^{15}N isotope signatures between plant species in the microcosms. Moreover, on the basis of the ^{15}N isotope signatures, we tested whether nitrogen transfer from legumes to nonlegumes occurred and whether this was influenced by rhizobia.

(3) How do nitrogen dynamics compare between microcosms and the natural field environment. We measured the natural abundance of ^{15}N in legumes and nonlegumes and compared the values with those in the microcosms (see Experiment 3).

Materials and methods

Experiment 1

The influence of rhizobia on plant community structure was tested using 16 replicate microcosms that simulated species-rich dune grassland. The plant species and bacterial isolates used in the microcosms all co-occurred in a dune grassland (Provinciale Waterleidingsduinen Noord Holland; Egmond Binnen; coordinates: 52°40′N, 4°39′E), which is referred to as the field site. Sixteen microcosms were established in $29 \times 21 \times 23$ cm containers. The containers were filled with 10 kg of autoclaved (110 °C for 2 h) nutrient-poor sand collected from the field site. In each container, 83 seedlings of 11 plant species were planted at random, and placed at regular distances (2.5 cm) from each other according to a predefined design. The number of seedlings planted per species (shown in parentheses below) corresponded approximately to their natural abundance in the field. The plant communities consisted of the following species: legumes: *Lotus corniculatus* L. (seven), *Ononis repens* L. (three), *Trifolium repens* L. (six); grasses: *Festuca ovina* L. (32), *Anthoxanthum odoratum* L. (seven), *Koeleria macrantha* (Ledeb.) Schultes (seven); forbs: *Plantago lanceolata* L. (six), *Hieracium pilosella* L. (six), *Achillea millefolium* L. (three), *Thymus pulegioides* L. (three), *Hypochaeris radicata* L. (three). The plant communities were established on 6–8 June 2002. Prior to the establishment of the microcosms, seeds were sterilized with 1% commercial bleach for 10 min, washed with sterilized water and placed on 1.6% water–agar plates (or 0.4% for the grasses). The hard-coated seeds of *O. repens* were incubated in concentrated sulphuric acid for 8 min prior to the bleaching step. Depending on the germination rate of each plant species, seedlings were incubated at 24 °C for 4–9 days and then transferred into the microcosms. Commercially available seeds of wild plants that originated from natural populations in The Netherlands were used (Cruydt-hoeck, Groningen, The Netherlands).

It is difficult to study the ecological function of rhizobia (and other bacteria) because they can easily disperse and cause contamination in control treatments. We minimized the chance of contamination by using containers with a controlled irrigation system, a large and modified Leonard

jar system (Leonard, 1943) and by watering the microcosms in a laminar flow chamber. Each container consisted of two compartments: a lower 'water' reservoir with a volume of 4.9 L and an upper soil reservoir of 9.2 L. The soil in the upper compartment was irrigated through wicks that extended into the water reservoir. All material was either autoclaved or sterilized with 70% alcohol in a laminar flow chamber prior to handling.

Half of the containers were inoculated with a water suspension of *Rhizobium* bacteria (the rhizobia treatment) and the remaining eight containers (the controls) were mock inoculated with an autoclaved suspension. Inoculum (60 mL) was injected into the soil at six spots that were equally distributed within the container. The inoculum used in this experiment contained a mixture of nine different strains that had been isolated from root nodules of *L. corniculatus* (three strains), *O. repens* (three strains) and *T. repens* (three strains) collected from the field site. From each strain, an equal volume of bacterial suspension with an optical density (OD) of 1.0 was used. The bacteria were isolated from the nodules and cultivated on solidified tryptone-yeast extract (TY)-agar plates (Beringer, 1974) according to standard methods (Somasegaran & Hoben, 1994). The strains were characterized by sequencing of the partial 16S rRNA with the general primers Y1 (Muyzer *et al.*, 1993) and R518 (Young *et al.*, 1991). Sequences of the three strains isolated from *T. repens* had their nearest match with 16S rRNA sequences from *Rhizobium leguminosarum* (accession numbers AY222325–AY222327). Two strains isolated from *L. corniculatus* and one from *O. repens* showed similarity to 16S rRNA sequences from *Mesorhizobium loti* (accession numbers AY222329, AY222330 and AY222332). Sequences of the remaining three strains had their nearest match to sequences from phyllobacteria (including *Rhizobium* species) and bacteria isolated from the rhizosphere (accession numbers AY222328, AY222331 and AY222333). The strains were also tested with regard to their ability to nodulate individual plants of *T. repens*, *L. corniculatus* and *O. repens* in Magenta jars using standard methods (Somasegaran & Hoben, 1994). Strains isolated from *T. repens* were host specific, in that they only induced root nodules on *T. repens* (Table 1). Strains isolated from *L. corniculatus* and *O. repens* induced root nodules on *L. corniculatus* and *O. repens*, but not on *T. repens* (Table 1). Two strains (AY222328 and AY222331) did not induce any root nodules.

The established microcosms were maintained in a climate room with day and night temperatures of 21 °C and 15 °C, respectively. Plants received a photon flux density of 375 μ mol m^{-2} s^{-1} and a day length of 16 h. The water reservoir was filled weekly with sterilized demineralized water, which was added to a tube positioned at one edge of the container. Handling and watering of the microcosms occurred in a laminar flow chamber. The 16 containers were randomized weekly. A modified low-nitrogen Hoagland nutrient solution was added to the communities five times, at weekly intervals, between 17 July and 23 August 2002. Each time, 20.6 mg of nitrogen and 8.28 mg of phosphorus were given. The total amounts of nitrogen and phosphorus given to each container were 62.5 kg N ha^{-1} $year^{-1}$ and 25 kg P ha^{-1} $year^{-1}$. The microcosms were harvested after 14 weeks on 18–20 September 2002. The above-ground biomass (dry weight) was determined for each plant species. The total root biomass (dry weight) and the number of flowers, or flower heads (in the case of *L. corniculatus*), were also determined. The roots of the plants were intermingled and it was not possible to determine the root biomass for individual plant species. Plant evenness was assessed using the equitability index (*J*) (Begon *et al.*, 1996). Equitability is a measure of the evenness with which species are distributed in a community.

Table 1. Nodulation capacities of bacterial isolates used in this study

	Plants nodulated		
Bacterial isolate*	*Trifolium repens*	*Lotus corniculatus*	*Ononis repens*
T.r 1 (AY222325)	+	−	−
T.r 2 (AY222326)	+	−	−
T.r 3 (AY222327)	+	−	−
L.c 1 (AY222328)	−	−	−
L.c 2 (AY222329)	−	+	+
L.c 3 (AY222330)	−	+	+
O.r 1 (AY222331)	−	−	−
O.r 2 (AY222332)	−	+	+
O.r 3 (AY222333)	−	+	+

*The bacterial isolates were isolated from root nodules of *Trifolium repens* (T.r), *Lotus corniculatus* (L.c) and *Ononis repens* (O.r). Accession numbers of each isolate are given in parentheses.
+, effective nodulation and nitrogen fixation; −, failure to nodulate.

The soil used in this experiment contained 308 mg of KCl-extractable nitrogen per container (nitrogen extracted as described in Houba *et al.* (1995). The soil was mixed with 3.85 mg of $K^{15}NO_3$ (98 at.% enriched), and the nutrient solution received 2.32 mg of $K^{15}NO_3$ to increase the relative abundance of ^{15}N. The relative abundance of plant-accessible ^{15}N in the microcosms was estimated to be 525‰ (as indicated by the broken line in Fig. 2, see later). This value is based on a mass balance of the amount of ^{15}N recovered in plant biomass (roots and shoots) in microcosms without rhizobia. The relative abundance of ^{15}N is commonly expressed in δ units, which denote the parts per thousand (‰) deviation from the $^{15}N/^{14}N$ ratio in atmospheric nitrogen, which is 0.0036765 and corresponds to 0.3663 at.% ^{15}N (Högberg, 1997). The nitrogen concentration (%) and $\delta^{15}N$ values of the shoots from each plant species and from the total roots were determined by continuous-flow

isotope ratio mass spectrometry [NC2500 Thermoquest Italia (Rodano, Italy) coupled to a ThermoQuest Finnigan Delta Plus Isotope Ratio Mass Spectrometer (Thermoquest Finnigan, Bremen, Germany)]. For each sample, 2–4 mg of plant material was analysed.

For legumes, the percentage of symbiotically fixed nitrogen was calculated as:

$$N_{fix}\,(\%) = [(N_r - N_{nr})/N_r] \times 100 \quad (1)$$

where N_r is the amount of nitrogen of a legume grown in a microcosm inoculated with rhizobia and N_{nr} is the amount of nitrogen of a legume grown in a control microcosm.

Experiment 2

Twenty-four microcosms simulating the field site were established as described for Experiment 1. The same plant species were used as in Experiment 1, with the exception of *H. radicata* and *Th. pulegioides* which were replaced by the two forbs *Silene vulgaris* (Moench) Garcke and *Erodium cicutarium* (L.) L'Hérit. In addition, a fourth legume, *Medicago lupulina* L., was included. The number of seedlings transferred into each microcosm at the beginning of the experiment is given in Table 2. The 80 individuals were planted at fixed distances (2.5 cm) from each other according to a predefined design. Twelve different planting designs were used, each being assigned to one replicate of each treatment. A randomized block design was employed (see below) in which each plant design constituted a block. Plants in microcosms belonging to one block thus had the same neighbours, whereas plants in different blocks had different neighbours. Twelve blocks (12 planting designs) were present. This approach was chosen to avoid the possibility of potential differences between treatments being confounded by neighbourhood interactions and initial plant species compositions.

Before planting, each microcosm was inoculated with a microbial community consisting of five fungal and bacterial strains that had been isolated from soil samples collected from the field site. The strains were isolated according to standard protocols. The soil material was suspended in water and an aliquot was transferred to malt agar (20 g malt extract, 3 g peptone and 16 g agar per litre, as described in Eger, 1976) and TY-agar (Beringer, 1974) plates to isolate fungi and bacteria, respectively. Five fungal and bacterial strains that varied in morphology were selected and propagated on malt agar and TY-agar, respectively. Each microcosm received three agar plugs per fungal strain. For each bacterial strain, a 2 mL bacterial suspension with $OD_{580} = 0.75$ was added to the microcosm. A pilot experiment showed that these bacteria did not induce nodules on the four legumes. These microbes were added to the microcosms to establish a microbial community and to enhance ecological relevance. Moreover, by adding these microbes, we intended to increase nutrient cycling in the communities, because many microbes contribute to the decomposition of plant material.

Table 2. Mean plant survival, mean shoot biomass and mean $\delta^{15}N$ values of 12 different plant species in grassland microcosms (Experiment 2)

	Plant species													
	Grasses			Legumes				Forbs						
Treatment	F.o	A.o	K.m	L.c	T.r	M.l	O.r	H.p	P.l	A.m	S.v	E.c	Roots	Total
No. of individuals	32	7	7	6	4	3	3	4	5	3	3	3		
Plant survival (%)														
+R	96	97	90	79	64	85	55	80	84	76	73	33		
−R	91	96	87	63	5	10	20	83	92	67	87	40		
P value	0.30	0.45	1.0	0.65	< **0.001**	**0.001**	0.02*	0.82	1.0	0.35	0.17	0.86		
Plant biomass ($g\,m^{-2}$)														
+R	134	203	14.6	410	196	41.9	159	1.31	36.4	1.97	23.8	10.3	2096	3344
−R	148	217	13.8	8	0.49	0.49	1.97	1.31	70.8	1.81	21.2	13.8	1132	1673
P value	0.79	0.69	0.27	< **0.001**	< **0.001**	< **0.001**	**0.005**	0.63	0.16	0.54	0.96	0.39	**0.003**	< **0.001**
$\delta^{15}N$ (‰)														
+R	53.6	64.9	48.0	–0.4	0.68	0.66	4.96	49.2	58.0	54.0	56.3	45.9	12.2	
−R	55.2	65.6	40.2	48.1	24.3	27.9	31.5	37.2	57.3	42.2	52.5	41.4	40.3	
P value	0.99	0.61	0.21	< **0.001**	**0.003**	0.006*	< **0.001**	0.03*	0.84	0.14	0.39	0.29	< **0.001**	

The microcosms were inoculated with rhizobia (+R) or left non-inoculated (−R). The number of seedlings planted in the microcosms is given in the first row. Root biomass and total biomass are also given. $\delta^{15}N$ values were obtained from above-ground plant material of each plant species at the second harvest. The $\delta^{15}N$ value from total root material is also given. *P* values for the corresponding analysis of variance (ANOVA) are given in the last row below each variable. *P* values in bold indicate a significant difference between the two treatments after a sequential Bonferroni *post hoc* test.

**P* values were significant when tested individually, but not after a sequential Bonferroni correction for multiple testing.

F.o, *Festuca ovina*; A.o, *Anthoxanthum odoratum*; K.m, *Koeleria macrantha*; L.c, *Lotus corniculatus*; T.r, *Trifolium repens*; M.l, *Medicago lupulina*; O.r, *Ononis repens*; H.p, *Hieracium pilosella*; P.l, *Plantago lanceolata*; A.m, *Achillea millefolium*; S.v, *Silene vulgaris*; E.c, *Erodium cicutarium*.

Microcosms inoculated with rhizobia contained one strain isolated from *T. repens* (accession number AY222326), one from *L. corniculatus* (accession number AY222329), one from *O. repens* (accession number AY222333) and one from *M. lupulina* (accession number AY883412 with a nearest match to *Sinorhizobium*). An inoculation experiment revealed that this isolate induced root nodules on *M. lupulina*. Details on the isolation, characterization and inoculation of the rhizobia are given above (Experiment 1).

The grassland microcosms were established in April 2003. The microcosms were fertilized at regular intervals with a low-nitrogen modified Hoagland solution (14 times), and received a total of 144 mg of phosphorus and 288 mg of nitrogen per container. The total amounts of nitrogen and phosphorus given to each container were 72 and 36 kg P ha^{-1} $year^{-1}$. The soil was mixed with 0.58 mg $K^{15}NO_3$ (98 at.% enriched), and the nutrient solution received a total of 0.61 mg $K^{15}NO_3$ to increase the relative abundance of ^{15}N. The $\delta^{15}N$ values obtained for Experiments 1 and 2 cannot be compared, as the concentration of the ^{15}N tracer in Experiment 2 was lower. To simulate grazing, plants were clipped after 180 days (3 cm above the soil surface). Harvested plant material was dried and weighed. Clipping was performed in a laminar flow chamber with scissors that were regularly sterilized to avoid contamination. After 240 days, the microcosms were completely harvested. Plants were cut at the soil surface, sorted by species and dried. For each plant species, the number of living individuals was recorded to determine plant survival. Plant roots were separated from soil, scored for the presence of root nodules, and the total root biomass (dry weight) was determined. The above-ground biomass (dry weight) was determined for each plant species by adding weights for harvests 1 and 2. Roots and shoots of each species were ground (two harvests separately) and the nitrogen concentration and $\delta^{15}N$ values were determined. Two microcosms from the control treatment were removed because they were contaminated with rhizobia (the legumes formed pink nodules and had nitrogen concentrations and $\delta^{15}N$ values typical for legumes with symbiotic nitrogen fixation). One microcosm in the rhizobial treatment was removed because most plants died after the experiment had been set up.

Experiment 3

The natural abundance of ^{15}N was determined for plant material harvested from the field site. The same plant species that were used for Experiment 1 were analysed in order to test whether plant species at the field site used the same nitrogen sources as those in the microcosms. Above-ground material of each plant species was collected at seven locations on 26 August 2002. The distance between neighbouring species at each location was maximally 15 cm. *Hypochaeris radicata* occurred at two locations and only two samples of this species were analysed. Plant material was dried and ground. The nitrogen concentration and $\delta^{15}N$ value of each plant species were measured using continuous-flow isotope ratio mass spectrometry (see above). The percentage of symbiotically fixed nitrogen was calculated as follows (Högberg, 1997):

$$N_{fix}\,(\%) = [(\delta^{15}N_{ref} - \delta^{15}N_{leg})/(\delta^{15}N_{ref} - B)] \times 100 \quad (2)$$

where $\delta^{15}N_{ref}$ is the average $\delta^{15}N$ value of the nonlegumes and $\delta^{15}N_{leg}$ is the $\delta^{15}N$ value of the legume in the field. B is the $\delta^{15}N$ value of the nitrogen-fixing legume when totally dependent on atmospheric nitrogen. In this study, B was assumed to be zero.

Statistical analysis

An analysis of variance (ANOVA) was performed on the variables studied to test whether microcosms inoculated with rhizobia varied from control microcosms without rhizobia (GLM; SPSS version 10.1, SPSS Inc., Chicago, IL). If necessary, variables were $\log_n$ transformed to meet the requirement of homoscedasticity of variance between both treatments. A nonparametric test (Wilcoxon's test) was performed when inequality of variances between the means of the treatments still occurred after the variables had been transformed. ANOVA consisted of one factor (with or without rhizobia) in Experiment 1 and two factors (rhizobia and block) in Experiment 2. The factor block consisted of 12 levels (12 replicates). For Experiment 2, the factor rhizobia was treated as a fixed effect and the factor block as a random effect. A significant block × rhizobia effect was not expected, and the rhizobia effect was tested using the residual mean square as denominator to calculate the F ratio (Newman *et al.*, 1997). The block effect was not significant for any of the variables tested. ANOVA (or the nonparametric equivalent) was performed for shoot biomass, shoot nitrogen concentration, amount of nitrogen and shoot $\delta^{15}N$ values for each plant species in Experiments 1 and 2. Such multiple testing of many plant species increases the chance of finding a significant result (Holm, 1979; Rice, 1989). A sequential Bonferroni analysis was therefore performed as a *post hoc* test to reduce the likelihood of increasing Type I errors (Holm, 1979; Rice, 1989).

Results

Experiment 1

Microcosms simulating dune grassland were established to study the impact of rhizobia on plant communities. The above-ground biomass was 34.6% higher in microcosms inoculated with rhizobia (Fig. 1a). This positive effect of rhizobia was mainly attributed to the influence of rhizobia

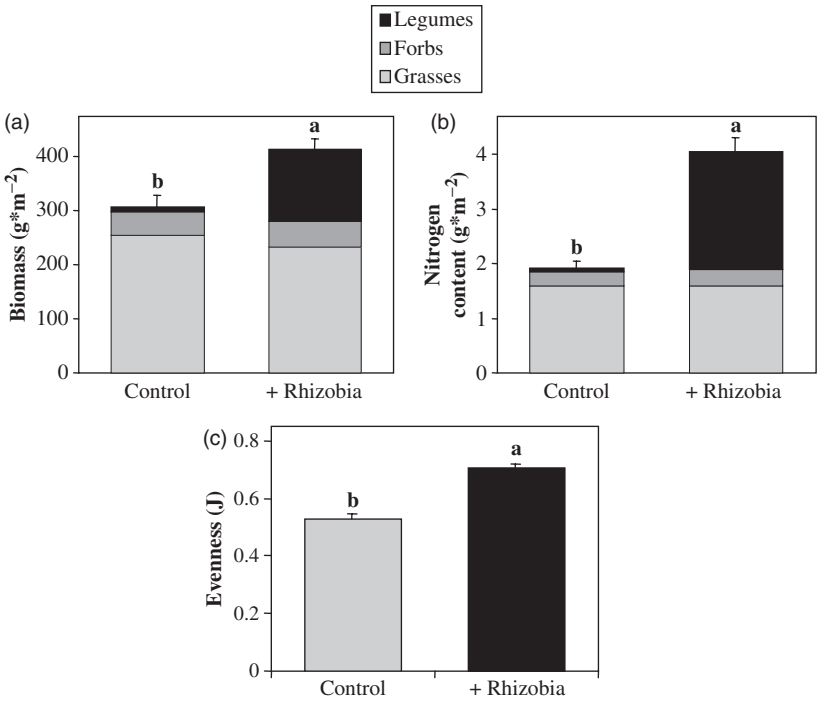

Fig. 1. Total above-ground biomass (a), total above-ground nitrogen (b) and evenness (c) (mean ± standard error) in microcosms simulating dune grassland (Experiment 1). Microcosms were inoculated with rhizobia or left noninoculated (control). Different letters above the columns indicate a significant difference for total above-ground biomass ($F_{1,14} = 11.1$; $P \leq 0.005$), total amount of above-ground nitrogen ($F_{1,14} = 58.0$; $P \leq 0.0001$) and evenness ($F_{1,14} = 58.8$; $P \leq 0.0001$) according to one-way analysis of variance. The biomass and the amount of nitrogen of the legumes differed significantly between the treatments according to a nonparametric Wilcoxon's test ($P \leq 0.001$). The biomass and the amount of nitrogen of grasses and forbs did not differ significantly between control microcosms and inoculated microcosms.

on legume growth. When plant communities were inoculated with rhizobia, the above-ground biomass of the three legumes *Lotus corniculatus*, *Ononis repens* and *Trifolium repens* increased strongly (16-, 14- and 13-fold, respectively). In contrast, the above-ground biomass produced by the eight nonlegumes, five forbs and three grass species was not influenced significantly by rhizobia. Accordingly, rhizobia altered the plant community structure by promoting plant productivity and by differentially influencing plant growth. The total root biomass did not vary significantly between the two treatments (1214 g m^{-2} without rhizobia; 1502 g m^{-2} with rhizobia). Moreover, *L. corniculatus* developed flowers in microcosms inoculated with rhizobia [on average, 24.8 flower heads; standard error (SE) = 5], whereas no flowers were observed in control microcosms. The roots of the three legumes in plant communities inoculated with rhizobia contained numerous pink-coloured nodules. In the control communities, no nodules were formed, indicating that we managed to prevent contamination.

The total amount of nitrogen in above-ground plant material increased by 85% when rhizobia were present (Fig. 1b). The amounts of nitrogen from whole microcosms (above-ground plus below-ground biomass) were 6.1 kg N m^{-2} in noninoculated and 11.8 kg N m^{-2} in inoculated communities. The majority of plant nitrogen was accumulated in roots (69% in control microcosms; 65% in inoculated microcosms). The amount of above-ground nitrogen of legumes growing in inoculated microcosms was 14.3 times higher than that in control microcosms. In contrast, the amount of nitrogen of grasses and forbs did not differ significantly between the two treatments (Fig. 1b).

The equitability index for evenness was 34.3% higher in communities inoculated with rhizobia than in noninoculated controls (Fig. 1c). This promoting effect of rhizobia on plant evenness disappeared when the biomass of the three legumes was excluded from the analysis (data not shown). This indicates that the positive effect of rhizobia on evenness is due to specific growth stimulation of the legume species. The number of species present in both treatments did not vary (11 species were planted at the beginning of the experiment and these species were still present at the time of harvest).

The nitrogen concentration (expressed as a percentage of dried biomass) of the three legume species almost doubled in microcosms inoculated with rhizobia (Fig. 2). The nitrogen concentration and the amount of nitrogen of the eight nonleguminous species did not vary between the two treatments (Fig. 2; data not shown). The $\delta^{15}N$ values of nonlegumes and legumes grown without rhizobia corresponded to the estimated $\delta^{15}N$ value of plant-available nitrogen in the soil (Fig. 2). This suggests that legumes and nonlegumes used the same nitrogen source when rhizobia were absent. In contrast, the $\delta^{15}N$ values of all legumes inoculated with rhizobia were significantly lower (Fig. 2), indicating that legumes obtained symbiotically fixed nitrogen. The percentage of symbiotically fixed nitrogen was 97% for each legume species [using Equation (1) given in the Materials and methods section]. With Equation (2) (using

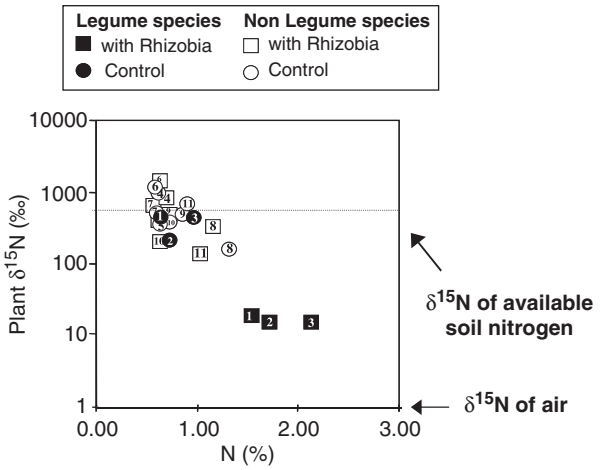

Fig. 2. Nitrogen concentrations (expressed as a percentage of shoot biomass) and abundance of ^{15}N ($\delta^{15}N$) of legumes and nonlegumes grown in microcosms (Experiment 1). Plants were inoculated with rhizobia or left noninoculated (controls). The microcosms contained three legume species (filled squares or filled circles) and eight nonlegume species (open squares or open circles). The $\delta^{15}N$ values of atmospheric nitrogen (standardized at 0‰) and available soil nitrogen ($\pm$ 525‰) are shown with arrows. Filled symbols relate to legumes and open symbols to nonlegumes. Numbers in symbols refer to the following plant species: 1, *Lotus corniculatus*; 2, *Trifolium repens*; 3, *Ononis repens*; 4, *Festuca ovina*; 5, *Anthoxanthum odoratum*; 6, *Koeleria macrantha*; 7, *Plantago lanceolata*; 8, *Hieracium pilosella*; 9, *Achillea millefolium*; 10, *Hypochaeris radicata*; 11, *Thymus pulegioides*.

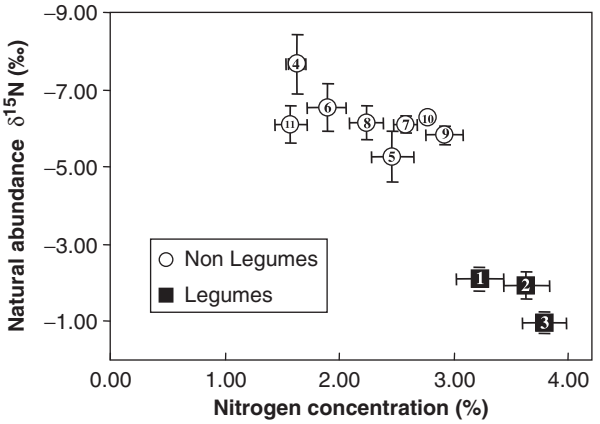

Fig. 3. Nitrogen concentrations (expressed as a percentage of biomass) and natural abundance of ^{15}N ($\delta^{15}N$) in legumes and nonlegumes harvested from the field site (Experiment 3). Means $\pm$ standard errors are shown. Numbers in symbols refer to different plant species (see Fig. 2).

the average $\delta^{15}N$ value of the eight nonlegume species as a reference), the amount of symbiotically fixed nitrogen was the same and was estimated to be 97% for each legume species.

Experiment 2

In a long-term experiment, the effect of rhizobia on plant communities was studied in the presence of other soil microbes. The plant survival of *Medicago lupulina* and *T. repens* was significantly higher in microcosms inoculated with rhizobia than in noninoculated controls (Table 2). When rhizobia where present, legume survival increased 2.9-fold (averaged across the four leguminous species). The survival of grasses (on average 93%) and forbs (on average 72%) was high and independent of the presence of rhizobia. The biomass of *L. corniculatus*, *M. lupulina*, *O. repens* and *T. repens* increased dramatically (51-, 85-, 81- and 398-fold, respectively) when microcosms were inoculated with rhizobia. As a result of enhanced legume growth, the total biomass in microcosms inoculated with rhizobia doubled compared with that in noninoculated controls. The biomass production of the nonlegumes was not affected by rhizobia. The $\delta^{15}N$ values of nonlegumes were similar in all microcosms when shoot material from the first harvest (data not shown) or final harvest was analysed (Table 2). The nitrogen concentration and the amount of nitrogen of the nonlegumes were also similar in all microcosms (data not shown). These results indicate that no nitrogen transfer from legume to nonlegume occurred during the experimental period of 8 months.

Experiment 3

Shoots of plant species used for Experiment 1 were collected from the field site, and their natural abundance of ^{15}N and nitrogen concentration were determined (Fig. 3). The $\delta^{15}N$ values of each of the three legumes (on average -1.7‰) were lower than those of the eight nonlegumes (on average -6.2‰). The nitrogen concentration of all leguminous species was also higher than that of nonlegumes (Fig. 3). The percentage of symbiotically fixed nitrogen in the field was estimated to be 66% for *L. corniculatus*, 89% for *O. repens* and 69% for *T. repens* (Equation (2); using the average $\delta^{15}N$ value of the eight nonlegume species as a reference).

Discussion

This paper demonstrates that nitrogen-fixing rhizobia promote plant evenness, productivity and nitrogen capture in legume-rich grassland microcosms. Previous studies have shown that mutualistic fungi play a key role in ecosystems by influencing plant diversity, plant abundance and ecosystem functioning (Grime *et al.*, 1987; van der Heijden *et al.*, 1998; Clay & Holah, 1999; Rudgers *et al.*, 2004). This study provides experimental evidence indicating that mutualistic bacteria have a large impact on plant community structure and functioning. The effects of rhizobia on plant biomass and plant community structure were largely a result of the

growth stimulation of the legumes, whereas the growth of nonlegumes was not influenced by rhizobia. Hence, the effects of rhizobia on vegetation structure are likely to be weak if legumes are absent. These data support the view that plant–microbe interactions are an integral part of ecosystems.

The observations also showed that the legume species studied required rhizobia to successfully coexist with nonlegumes. Rhizobia more than doubled the survival rates for three of the four legume species and had a large effect on the legume biomass and amount of nitrogen. The biomass and amount of nitrogen of the other plant species were not affected by rhizobia. This indicates that, in the presence of rhizobia, competitive interactions between legumes and nonlegumes did not occur. Instead, niche separation and resource partitioning were observed. Legumes utilized symbiotically fixed atmospheric nitrogen (over 90% of nitrogen was derived from rhizobia), whereas nonlegumes obtained nitrogen from the soil. Legumes and nonlegumes in the field also seemed to use different nitrogen sources. The relative abundance of the nitrogen isotope ^{15}N, the $\delta^{15}N$ value, in legumes collected from the field was close to zero, the signature of atmospheric nitrogen. This indicates that legumes in the field also rely on symbiotically fixed atmospheric nitrogen. In contrast, the $\delta^{15}N$ signatures of nonlegumes were negative (Fig. 3), indicating that nonlegumes acquired nitrogen from a different source (the soil). Other field studies have also found that legumes and nonlegumes have different $\delta^{15}N$ signatures (Högberg, 1997; Mulder *et al.*, 2002; Spehn *et al.*, 2002). Fractionation of nitrogen sources occurs in the field and this can lead to negative values for soil-available nitrogen (Högberg, 1997). This may explain why the nonlegumes at our field site showed negative $\delta^{15}N$ values. Importantly, the $\delta^{15}N$ values of plants in the field (Fig. 3) reflect natural abundances, and these values cannot be compared with the $\delta^{15}N$ values in the microcosms (Fig. 2; Table 2) where ^{15}N tracer was added.

The combination of rhizobial strains used in our experiments (all rhizobia were isolated from dune grassland) was efficient in promoting legume growth. This contrasts with observations from some agricultural systems in which ineffective rhizobia occur that do not necessarily promote legume performance (Denton *et al.*, 2000; Kiers *et al.*, 2002). Certain rhizobia exhibit a high degree of host specificity, whereas others associate with a wider range of legumes (Table 1) (Perret *et al.*, 2000). For instance, *Trifolium repens* and *Lotus corniculatus* plants in the field were infected by different rhizobia (Scheublin & van der Heijden 2001, unpubl. results). This suggests that the presence of a diverse rhizobial community is required for these legumes to grow and coexist. For this reason, we inoculated the microcosms with different rhizobial strains that were isolated from different host plants collected from the field site.

Several studies have shown that legumes increase nitrogen availability in the soil and that neighbouring plants obtain symbiotically fixed nitrogen from legumes via root exudates, decaying legume roots or mycorrhizal hyphal networks that transfer nitrogen from legumes to nonlegumes (Heichel, 1987; Haystead *et al.*, 1988; Simard *et al.*, 2002). The transfer of nitrogen from legumes to nonlegumes has been shown to occur as early as 25 days after nitrogen assimilation by legumes (Hogh-Jensen & Schjoerring, 2000), and can be stimulated by clipping shoots (Trannin *et al.*, 2000). Our data did not show nitrogen transfer from legumes to nonlegumes, even though Experiment 2 was maintained for 8 months and plants were clipped. It is worth noting in this context that mycorrhizal fungi, soil fauna and herbivores were absent in our experiments. These organisms are known to stimulate nitrogen cycling, and it would be of interest to test whether nitrogen transfer occurs when they are present. Such studies are in progress and should help to increase our understanding of how complex interactions in the rhizosphere shape plant communities.

Conclusions

Several studies have shown that symbiotic soil fungi act as regulators of plant productivity and diversity (Grime *et al.*, 1987; van der Heijden *et al.*, 1998). Our results add a new dimension to these observations by revealing the mechanism by which plants and symbiotic soil bacteria jointly act as drivers of plant productivity, nitrogen nutrition and plant community structure in legume-rich dune grassland. Moreover, our study indicates that the investigated legume species require rhizobia to successfully coexist with other plants in natural communities.

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